Activation tagging of ADR2 conveys a spreading lesion phenotype and resistance to biotrophic pathogens

Activation tagging of *ADR2* conveys a spreading lesion phenotype and resistance to biotrophic pathogens

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Summary

• An *Arabidopsis PR1::luciferase* (*LUC*) transgenic line was transformed with activation T-DNA tags and the resulting population screened for dominant gain-of-function mutants exhibiting constitutive LUC activity.

• LUC imaging identified *activated disease resistance 2* (*adr2*), which exhibited slowly spreading lesions in the absence of pathogen challenge. Molecular, genetic and histochemical analysis was employed to characterize this mutant in detail.

• *adr2* plants constitutively expressed defence-related and antioxidant genes. Moreover, this line accrued increased quantities of salicylic acid (SA) and exhibited heightened mitogen-activated protein kinase activity. *adr2* plants exhibited increased resistance against numerous biotrophic but not necrotrophic pathogens. The *adr2* phenotype resulted from the overexpression of a *Toll interleukin receptor* (*TIR*) *nucleotide binding site* (*NBS*) *leucine rich repeat* (*LRR*) gene (*At1g56510*). Constitutive *PR1* expression was completely abolished in *adr2 nahG*, *adr2 npr1* and *adr2 eds1* double mutants. Furthermore, heightened resistance against *Hyaloperonospora arabidopsis* Noco2 was compromised in *adr2 nahG* and *adr2 eds1* double mutants but not in *adr2 npr1*, *adr2 coi1* or *adr2 etr1* plants.

• These data imply that *adr2*-mediated resistance operates through an Enhanced Disease Susceptibility (EDS) and SA-dependent defence signalling network which functions independently from COI1 or ETR1.

Introduction

Plants have evolved an array of sophisticated mechanisms to detect and respond to attempted pathogen ingress. Preformed physical and chemical barriers constitute the first line of defence (Haralampidis *et al.*, 2001) upon which are superimposed a battery of inducible defence responses (Yun *et al.*, 2003; Nürnberger & Lipka, 2005). Prominent in the triggering of these protective mechanisms is a repertoire of resistance (*R*) gene products, which recognize either directly or indirectly pathogen effector proteins (Dangl & Jones, 2001). An effector protein whose presence is detected by a resistant plant is termed an AVIRULENCE (AVR) gene product although its likely function is to aid pathogenesis on a susceptible host (Nomura *et al.*, 2006). The largest class of *R* genes encode NUCLEOTIDE BINDING SITE (NBS) LEUCINE RICH REPEAT (LRR) proteins, which can be divided into subgroups defined by the presence of either COILED-COIL (CC) or TOLL INTERLEUKIN RECEPTOR (TIR) domains in their *N*-termini (Meyers *et al.*, 2003).

R gene-dependent pathogen recognition is usually associated with a form of genetically programmed plant cell death termed the hypersensitive response (HR) surrounding the site of attempted infection (Greenberg, 1997; Gilchrist, 1998). This cell death event has been shown to induce a phenomenon known as systemic acquired resistance (SAR) which establishes relatively durable, broad-spectrum protection from ordinarily virulent pathogens, throughout the plant (Durrant & Dong, 2004; Grant & Loake, 2007). The development of SAR correlates with a notable rise in peroxidase activity, increased lignin deposition and the expression of *PATHOGENESIS-RELATED* (*PR*) genes (Ryals *et al.*, 1996).

One of the most rapid biochemical changes engaged following pathogen recognition is the so-called oxidative burst

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producing reactive oxygen intermediates (ROIs), primarily superoxide (O_2^-) and hydrogen peroxide (H_2O_2), at the site of attempted invasion (Lamb & Dixon, 1997; Bolwell, 1999; Grant & Loake, 2000). These may serve both as antimicrobial agents and signalling molecules. Nitric oxide (NO), (Delledonne *et al.*, 1998) and related S-nitrosothiols (SNOs) (Feechan *et al.*, 2005; Hong *et al.*, 2007; Wang *et al.*, 2009) also function as important signals in plant disease resistance against pathogens and may interact with ROIs to mediate the HR (Delledonne *et al.*, 2001). Salicylic acid (SA) accumulates in plant tissue responding to pathogen infection and is essential for the induction of SAR as well as being required for some *R* gene-mediated responses (Delaney *et al.*, 1994). Currently, the accumulating data supports a role in which SA acts at multiple nodes in the defence signalling network, possibly by functioning as a signal amplifier (Fauth *et al.*, 1996; Mur *et al.*, 1996; Shirasu *et al.*, 1997). Accumulating evidence suggests that the balance and cooperation between NO, ROI and SA produced early in the plant resistance response is required for the full expression of the HR (Kumar & Klessig, 2000; Delledonne *et al.*, 2001).

A number of *Arabidopsis* mutants have been described that exhibit constitutive activation of *PR* gene expression and resistance to biotrophic pathogens, such as *cim*, *cpr*, *cir1*, *cep* and *dnd1* mutants (Bowling *et al.*, 1994; Clarke *et al.*, 1998; Yu *et al.*, 1998; Silva *et al.*, 1999; Maleck *et al.*, 2002; Murray *et al.*, 2002). In other mutants, including *cpr5*, *adr1*, *hrl1*, *lsd* and *acd* (Dietrich *et al.*, 1994; Greenberg, 1994; Bowling *et al.*, 1997; Rate *et al.*, 1999; Devadas *et al.*, 2002; Grant *et al.*, 2003) these traits are also associated with the spontaneous development of HR-like cell death lesions. Another related mutant, *lsd1*, exhibits normal HR after infection by various incompatible pathogens, but runaway cell death (RCD) is initiated subsequently at the margins of these sites (Dietrich *et al.*, 1994).

Activation tagging has successfully been employed to identify novel defence signalling components in *Arabidopsis* (Grant *et al.*, 2003; Tani *et al.*, 2004; Nurmberg *et al.*, 2006). We have carried out an activation tagging approach in a designer *PR1*::*LUCIFERASE* (*LUC*) genetic background (Murray *et al.*, 2002; Grant *et al.*, 2003) to further uncover genes whose enhanced expression leads to the establishment of broad-spectrum disease resistance against virulent pathogens. In this report, we describe the characterization of *adr2*, which exhibits a spreading cell death phenotype and broad-spectrum resistance against biotrophic pathogens. Furthermore, our findings show that the *adr2* phenotype results from over-expression of a *TIR NBS LRR* gene.

Materials and Methods

Plant growth

Arabidopsis thaliana (L.) Heynh. plants were placed in a phytochamber under short daylength conditions (8 h light, 16 h dark at 20°C) at a light intensity of 150 μmol m^{-2} s^{-1}. Plants destined for *Agrobacterium* transformation, were placed under long daylength conditions (16 h light, 8 h dark) at a high light intensity of 15 000 lux, 90 W m^{-2} or low intensity 3000 lux, 14 W m^{-2}. All chemicals used were purchased from Sigma-Aldrich unless stated otherwise. Experiments were routinely undertaken with 23-d-old plants during the lesion initiation stage.

Activation tagging and LUC imaging

T-DNA activation tagging and generation of transgenic *Arabidopsis* plants were essentially carried out as described by Grant *et al.* (2003) with transformation via the floral dip procedure (Clough & Bent, 1998). The LUC imaging was performed as described (Grant *et al.*, 2000).

In vitro LUC activity

Measurement of *in vitro* LUC activity was carried out using LUC Assay System kit (Promega) according to the manufacturer's instructions. A microplate luminometer EG&G Micro-Lumat LB96P (Berthold, London, UK) was used and LUC activity expressed as relative light units (RLU) over a 0.5 s time-period. For specific LUC activity, the protein concentration of each sample was determined by Bradford Micro-Assay (Bio-Rad), using BSA as protein standard (Bradford, 1976). Specific LUC activity was calculated as RLU μg^{-1} total protein.

Histochemistry and northern blot analysis

Trypan blue staining of leaves, for the examination of micro-lesions or fungal hyphae and spores, was carried out according to Bowling *et al.* (1997). *In situ* H_2O_2 detection, in epidermal leaf tissue, was performed using 3,3′-diaminobenzidine (DAB; Sigma) according to the protocol of Thordal-Christensen *et al.* (1997). Leaf autofluorescence was examined under UV illumination using UV epifluorescence microscopy (excitation filter 365 nm, dichroic mirror, 395 nm and barrier filter 420 nm; Dietrich *et al.*, 1994). In all cases, treated leaves were mounted on glass slides in 70% glycerol, subsequently examined using Leica Wild M3C microscope, and photographed. Northern analysis was undertaken as described by Chini & Loake (2005).

Biochemical analysis

A pyrogallol-based method was conducted to measure peroxidase (POD) activity (Kwak *et al.*, 1995). Catalase (CAT) activity was assayed according to the method of Abei (1984). In-gel kinase assay was essentially conducted according to Romeis *et al.* (1999). Leaf SA measurements were performed in 5-wk-old plants using a microscale high-pressure liquid chromatography (HPLC)-based procedure as described by Aboul-Soud *et al.* (2004). Total chlorophyll content was determined in 5-wk-old plants according to Lichtenthaler (1987).

Pathogen inoculations

The bacterial pathogen *Pst*DC3000 was grown and maintained as described by Whalen *et al.* (1991). Fungal and oomycete infection assays were performed according to Grant *et al.* (2003).

Mapping and cloning of *ADR2*

The homozygous *adr2* mutant, a Col-0 background carrying the *PR1::LUC* transgene, was crossed to L*er*. Next, 50 F_2 plants showing characteristic *adr2* HR-like lesions were used to obtain a rough map position for the wild-type *ADR2* gene by bulked segregant analysis (Lukowitz *et al.*, 2000). This was carried out using standard simple sequence-length polymorphism (SSLP) (Bell & Ecker, 1994) markers as reported on the *Arabidopsis* Information Resource database website (http://www.arabidopsis.org/). The position of the T-DNA activation tag within *adr2* plants was determined by thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) (Singer & Burke, 2003).

Results

Identification of *adr2* by activation tagging

A population of *c.* 9000 activation tagged plants which contained a *PR1*::*LUC* transgene were screened for enhanced LUC activity in the absence of pathogen challenge using an ultra low light imaging (Murray *et al.*, 2002; Grant *et al.*, 2003). The *activated disease resistance 2* (*adr2*) mutant line had sustained high levels of *PR1::LUC* expression throughout development. The LUC activity was particularly strong in cotyledons and all true leaves (Fig. 1a). Furthermore, *in vitro* enzymatic assays confirmed that *adr2* plants constitutively expressed significant LUC activity (Fig. 1b).

Phenotypic and biochemical characterisation of *adr2* plants

The *adr2* line is significantly smaller compared with wild-type Col-0 plants, displaying reduced stature and the development of macroscopic HR-like lesions (Fig. 2a). Growth of this mutant under sterile conditions confirmed that the appearance of these lesions is both stress- and pathogen-independent. Lesion development was more pronounced on homozygous compared with heterozygous *adr2* plants (Fig. 2b). The lesion distribution pattern was found to be uniform, emerging first at the tip of the leaf and progressing gradually towards the leaf origin, ultimately covering the total leaf surface, resulting in a mosaic-like appearance. At *c.* 6 wk postgermination these lesions had coalesced consuming the whole of older leaves (Fig. 2c).

Lesion development in both *adr2 adr2* and *adr2 ADR2* plants was not dictated by daylength. Microscopic analysis, performed on wild-type and *adr2 adr2* leaves by Trypan blue staining, revealed an absence of HR-like lesions in wild-type plants while in *adr2 adr2* plants macroscopic HR-like lesions were predominantly formed of clusters of dead cells that were spread over the leaf surface (Fig. 2d,e). As ROIs are thought to help cue lesion development (Grant *et al.*, 2000; Delledonne *et al.*, 2001), we also examined ROI accumulation by the DAB peroxidase-based method (Thordal-Christensen *et al.*, 1997). While DAB staining was largely absent in wild-type Col-0 plants (Fig. 2f), *adr2 adr2* leaves accumulated lesion-localized H_2O_2 (Fig. 2g).

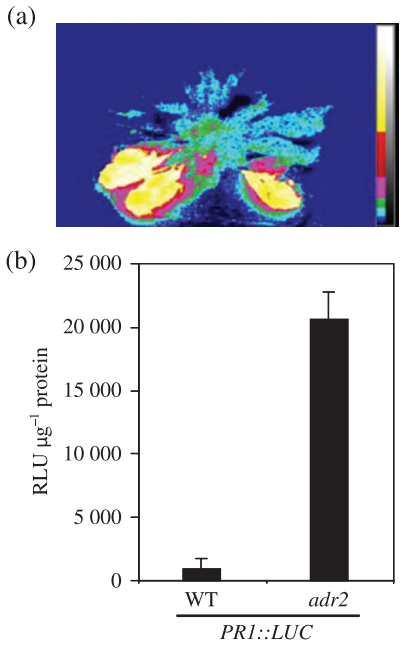

Fig. 1 *In planta* luciferase (LUC) imaging and *in vitro* LUC activity in *adr2* leaves. (a) A pseudo-coloured image of a homozygous *adr2* plant exhibiting bioluminescent leaves, indicative of high LUC activity. Each of the three leaves was treated with luciferin before imaging. The colour index to the right of the image denotes light intensity, where light colours are high and dark colours are low. (b) Graph depicting *in vitro* LUC activity in *adr2* leaves compared with *PR-1::LUC* control. Each data point represents the mean of LUC activity measured in three independent experiments. Error bars represent the standard error of the mean, $n = 3$.

Although UV fluorescence was largely absent from wild-type leaves that had first been cleared of their chlorophyll (Fig. 2h), it could be detected following challenge with *Pseudomonas syringae* pv. *tomato* (*Pst*DC3000) expressing the *avrB* avirulence gene (Bisgrove *et al.*, 1994; Grant *et al.*, 1995) (Fig. 2i). The accumulation of autofluorescent material in *adr2 adr2* plants appeared significantly stronger than that which had accrued in an HR-lesion triggered following recognition *Pst*DC3000(*avrB*) and was mainly confined to HR-like lesion sites (Fig. 2j). Hence, the accumulation of phenolic compounds in *adr2 adr2* leaves appeared to be correlated with the exhibition of cell death.

Increases in the local concentrations of salicylic acid (SA) underpin the establishment of basal disease resistance against

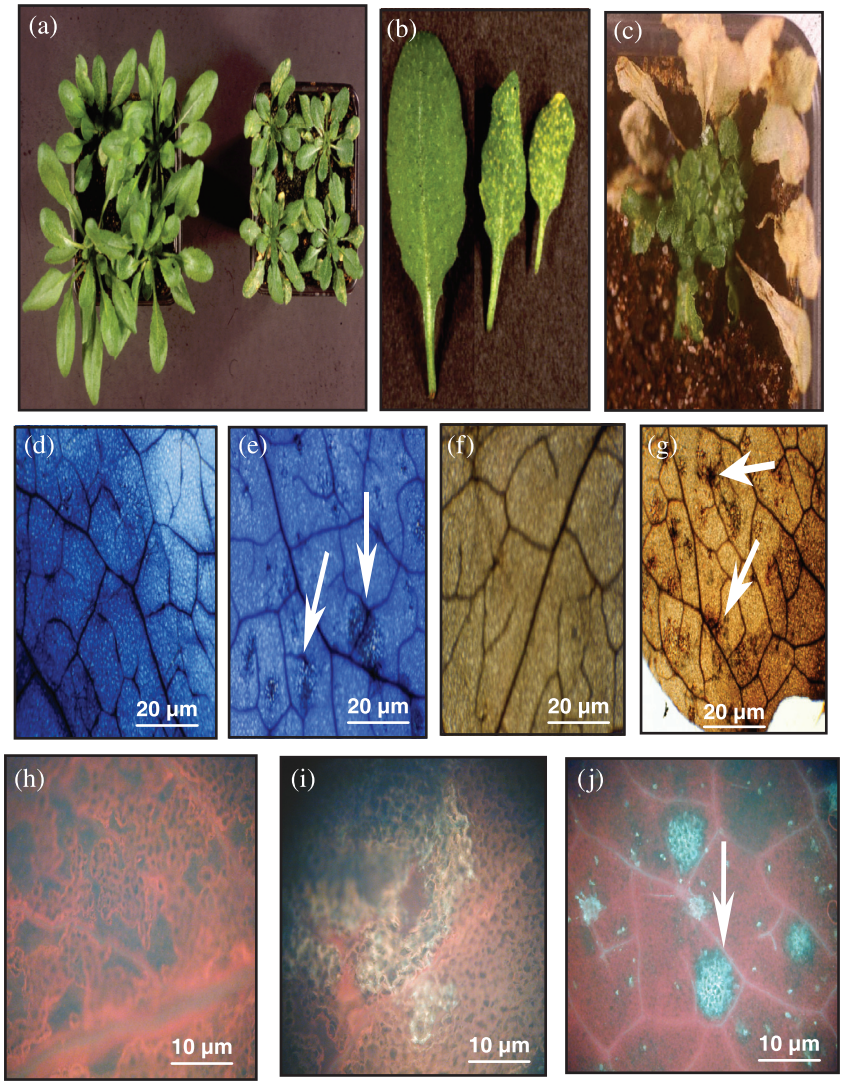

Fig. 2 Phenotypic and histochemical characterization of *adr2* plants. (a) Homozygous *adr2* plants (right) are reduced in stature compared with wild-type Col-0 plants (left). (b) *adr2* leaves show development of hypersensitive response (HR)-like lesions which are more extensive in homozygous (right) than in heterozygous *adr2* leaves (middle). Lesions are absent in wild-type plants (left). (c) *adr2* plants exhibit a progressive cell death phenotype that gradually spreads to consume the whole leaf by *c.* 6 wk postgermination. (d,e) Trypan blue (TB) staining of wild-type and *adr2* leaves. The TB stains leaf veins and dead cells, revealing the region with HR-like lesions. Note the pattern of clustered dead cells that is absent from wild-type leaves (d) and present in *adr2* leaves (e), revealed by arrows. (f,g) Hydrogen peroxide (H_2O_2) detection by the peroxidase-based 3,3′-diaminobenzidine (DAB)-uptake method. Leaves of *adr2* plants (g) were found to accumulate high levels of DAB staining (arrows) compared with wild type (f). (h) Wild-type plants show no UV autofluorescent material which marks the accumulation of phenolic compounds. (i) Wild-type leaf challenged with *Pst*DC3000(*avrB*) at 30 h postinoculation, showing an increase in autofluorescent material. (j) Leaves of *adr2* plants accumulate autofluorescent phenolic compounds in the absence of pathogens (illustrated by arrow). Experiments were repeated twice with similar results.

microbial pathogens (Delaney *et al.*, 1994). We therefore investigated whether SA and its glucoside conjugate (SAG) accumulated in *adr2 adr2* plants and an *adr2 adr2 nahG* double mutant. The *nahG* transgene encodes a salicylate hydroxylase which depletes cellular SA levels (Delaney *et al.*, 1994). Leaves of *adr2 adr2* plants were found to accumulate *c.* 8 times and 15 times more SA and SAG, respectively, than wild-type Col-0 (Fig. 3a,b). This result is consistent with reports of other mutants, which show spontaneous accumulation of SA and constitutive expression of *PR* genes (Durrant & Dong, 2004; Grant & Loake, 2007). Moreover, 95% of SA in *adr2 adr2* plants was present as SAG and accumulation of both SA and SAG was significantly reduced in *adr2 adr2 nahG* plants compared with the *adr2 adr2* line (Fig. 3a,b).

Quantification of soluble POD activity in crude protein extracts of *adr2 adr2* compared with wild-type Col-0 plants revealed a significant difference. Interestingly, *adr2 adr2* leaves exhibited *c.* 14 times more soluble POD activity than wild-type (Fig. 3c). Moreover, the activity of ionically bound POD was *c.* 10 times higher in *adr2 adr2* plants compared with that of wild type (Fig. 3c). Catalase was another antioxidant enzyme whose activity was examined in *adr2 adr2* plants. Surprisingly, there was no significant difference of either *in vitro* or in-gel CAT activity between *adr2 adr2* plant extracts compared with wild-type (data not shown).

Mitogen-activated protein kinase (MAPK) activity has been shown to be elevated in response to ROI accumulation following the oxidative burst triggered by attempted pathogen infection (Grant *et al.*, 2000). We therefore examined whether the accumulated H_2O_2 present in the *adr2 adr2* mutant could cue a similar profile of MAPK activity. In this context, *adr2 adr2* protein extracts were found to contain elevated MAPK activity, as determined by in-gel kinase assays (Fig. 3d). Two conspicuous myelin-basic protein (MBP)-phosphorylating activities of 46 kDa and 48 kDa were detected in extracts from *adr2 adr2* plants. By contrast, there was little MAPK-related activity detected in wild-type plants. Moreover, the profile of *adr2 adr2* MAPK activity could be mimicked by infiltrating wild-type

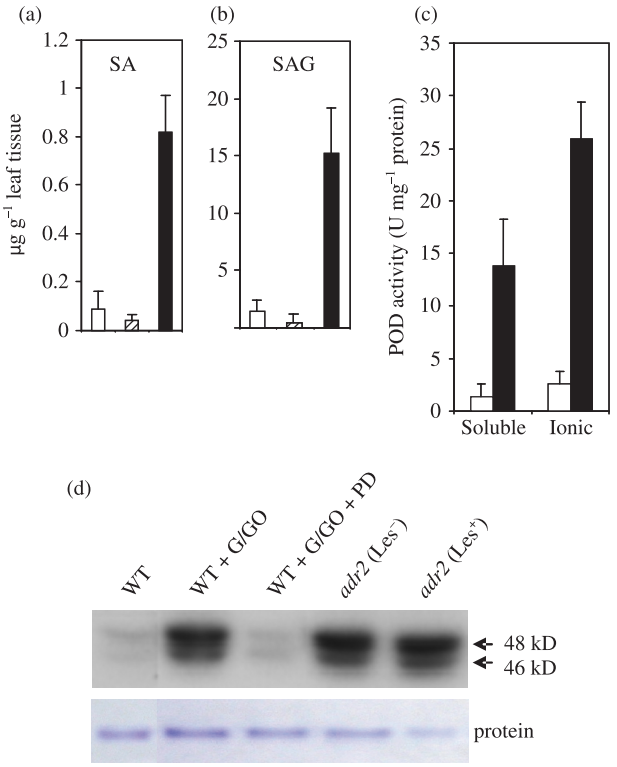

Fig. 3 Analysis of defence-related biochemical markers in *adr2* plants. (a) Quantification of the level of endogenous salicylic acid (SA) in wild-type (open bars), *adr2 adr2 nahG* (hatched bars) and homozygous *adr2* (closed bars) plants. (b) Quantification of the amount of endogenous salicylic acid glucoside (SAG) in wild-type, *adr2 adr2 nahG* and homozygous *adr2* plants. Each data point represents the mean of two independent samples; repeated experiments produced similar results. Error bars represent the standard error of the mean. (c) Soluble and ionically bound peroxidase (POD) activity in homozygous *adr2* (closed bars) and wild-type (open bars) plants. Each data point represents the mean of three independent POD activity measurements from three different plants. The experiment was repeated twice with similar results. The POD activity is expressed as U mg^{-1} total leaf protein. Error bars represent the standard error of the mean. (d) In-gel mitogen-activated protein kinase (MAPK) assay performed on extracts from the given plant genotypes. The leaves of some plants were treated with the hydrogen peroxide (H_2O_2)-generating system glucose/glucose oxidase (G/GO) (250 mM G/250 U ml^{-1} GO) and/or PD98059 (denoted as PD) a potent MAPK inhibitor in plants. Samples were collected 15 min post treatment. *adr2* (Les^-) and *adr2* (Les^+) were leaf samples from *adr2* plants either displaying or that had not yet developed macroscopic lesions, respectively.

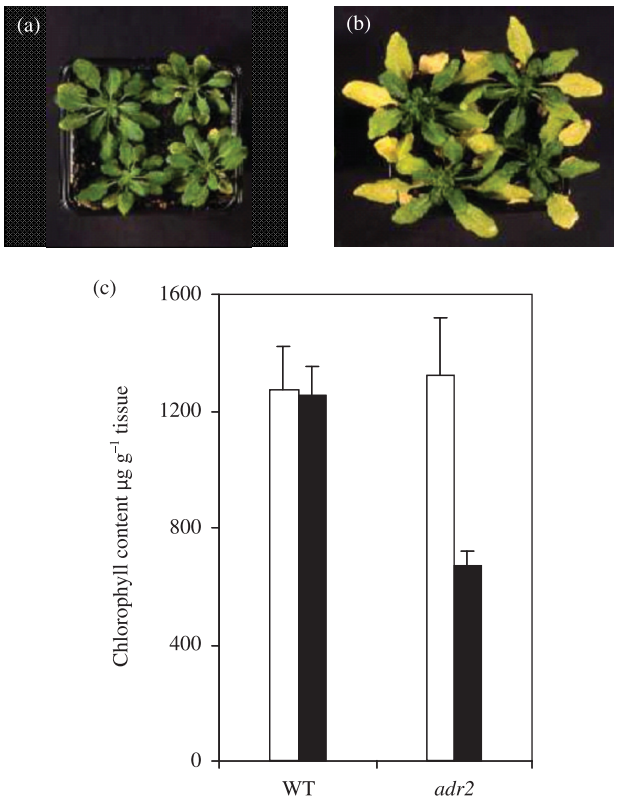

Fig. 4 Quantification of chlorophyll content in *adr2* and wild-type plants in response to changes in light intensity. (a) No chlorotic leaves are observed on homozygous *adr2* plants that were grown under constant low light intensity (LLI). (b) Extensive yellow lesion development occurred on *adr2* plants grown under high light intensity (HLI) for 14 d and then transferred to LLI. In some cases, lesion development covered most of the leaf surface. (c) Changes in chlorophyll content in homozygous *adr2* plants compared with wild type in response to either LLI (open bars) or HLI for first 14 d (closed bars) and then transferred to LLI. Chlorophyll content is represented as total chlorophyll per µg fresh leaf tissue. The experiment was repeated twice with similar results ($n = 3$). Error bars represent the standard error of the mean.

leaves with an H_2O_2-generating solution containing glucose/glucose oxidase (G/GO) within 15 min post infiltration (Fig. 3d). Furthermore, the MAPK activity induced by H_2O_2 could be blocked by co-infiltrating PD98059, a well-known mammalian MAPK inhibitor (Fig. 3d) (Grant *et al.*, 2000) confirming the source of the MBP-phosphorylating activity.

Homozygous *adr2* plants grown under low light intensity (LLI) did not go on to develop macroscopic HR-like lesions (Fig. 4a). By contrast, homozygous *adr2* plants grown under either continuous high light intensity (HLI) or HLI for 14 d and then subsequently shifted to low light intensity (LLI) subsequently developed prominent yellow lesions that coalesced to cover most of the leaf surface (Fig. 4b). We next examined the effect of changes in light intensity on chlorophyll content in *adr2 adr2* plants compared with wild type. A 50% reduction in chlorophyll content was observed in *adr2 adr2* plants that were first grown under HLI and then shifted to LLI (Fig. 4c). Interestingly, wild-type or *adr2 adr2* plants that were kept under LLI conditions had similar chlorophyll content (data not shown). Moreover, wild-type plants grown under HLI then shifted to LLI displayed no visible changes in chlorophyll content (data not shown). Thus, lesion development resulting in chlorophyll degradation in *adr2* plants was triggered in response high light intensity.

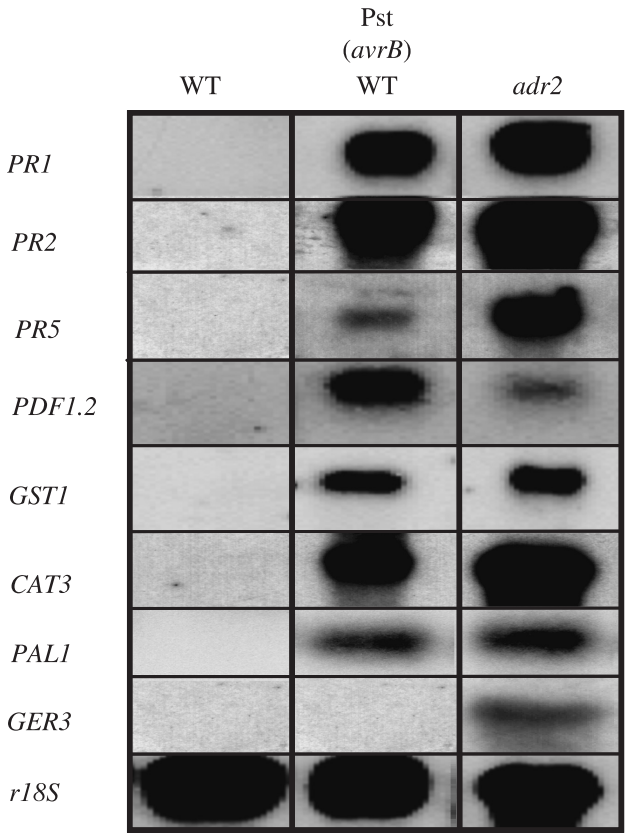

Fig. 5 Northern analysis of salicylic acid (SA)-, jasmonic acid/ethylene (JA/ET)- and antioxidant-related genes in *adr2* plants. Analysis of *PR-1*, *PR-2*, *PR-5*, *PDF1.2*, *GST1*, *CAT3*, *PAL1* and *GER3* transcripts in unchallenged and *Pst*DC3000(*avrB*) challenged plants and the *adr2 adr2* mutant line. Total RNA was extracted 24 h postinoculation (hpi) of *Pst*DC3000(*avrB*) for all genes except *PAL1*, where it was extracted at 2 hpi. The constitutively expressed gene *r18s* was used as an RNA equal loading and transfer control.

adr2 conveys constitutive expression of defence and antioxidant-related genes

Leaves of homozygous *adr2* plants were found to accumulate *PR1* transcripts, as revealed by northern blot analysis (Fig. 5), thus confirming previous LUC activity data (Fig. 1b). Furthermore, other SA-dependent genes including *PR-2*, *PR-5*, *GER3* and *PAL1* (Davis *et al.*, 1991; Ryals *et al.*, 1996; Berna & Bernier, 1999) were also activated, however, *PR-5* was induced to a greater extent in homozygous *adr2* plants compared with wild-type challenged with *Pst*DC3000(*avrB*) (Fig. 5). The ROI responsive gene *GST1* was also induced (Grant *et al.*, 2000). By contrast, the jasmonic acid (JA)/ethylene (ET)-regulated gene *PDF1.2* (Penninckx *et al.*, 1996) was not strongly expressed in the homozygous *adr2* line compared with wild-type plants challenged with *Pst*DC3000(*avrB*) (Fig. 5). Another antioxidant gene, *CAT3* (McClung, 1997), was also expressed in homozygous *adr2* plants.

adr2 plants exhibit broad-spectrum resistance against biotrophic but not necrotrophic pathogens

As homozygous *adr2* plants accumulate the key defence signalling molecule SA and display constitutive expression of numerous defence genes, we examined whether this line displayed resistance against usually virulent microbial pathogens. Therefore, we challenged these plants with one hemi-biotrophic and two biotrophic pathogens: the bacterium *Pst*DC3000 (Whalen *et al.*, 1991), the oomycete pathogen *Hyaloperonospora arabidopsis* Noco2, formerly *H. arabidopsis* (Parker *et al.*, 1996) and the fungal pathogen *Golovinomyces cichoracearum* UED1 (Yun *et al.*, 2003), respectively. We also challenged *adr2 adr2* plants with the necrotrophic fungal pathogen, *Botrytis cinerea* (Nurmberg *et al.*, 2006).

The homozygous *adr2* plants exhibited conspicuous resistance against the virulent pathogen *Pst*DC3000 (Fig. 6a). Bacterial growth in this line was approx. 0.5 log less than that present in wild-type Col-0 plants 3-d postinoculation (dpi) (Fig. 6a). Moreover, the growth of *H. arabidopsis* Noco2 was also significantly suppressed in *adr2 adr2* leaves compared with wild-type at 10 dpi (Fig. 6b). Interestingly, the level of resistance exhibited by homozygous *adr2* leaves mirrored that of wild-type leaves treated with SA (Fig. 6b). As expected, *nahG* plants exhibited increased susceptibility to this pathogen (Delaney *et al.*, 1994). Furthermore, *adr2 adr2* leaves also displayed increased basal resistance against *G. cichoracearum* UED1 (Fig. 6c) and this observation was confirmed by Trypan blue staining (Fig. 6c). By contrast, the response of homozygous *adr2* plants to *Botrytis cinerea* was indistinguishable from wild-type (data not shown).

Characterization of defence responses in *adr2* double mutants

To further investigate the individual role of known defence signalling pathways to the establishment of the *adr2* phenotype, we also crossed *adr2 adr2* plants with the *coi1* mutant, which is insensitive to JA (Feys *et al.*, 1994), the ET insensitive mutant *etr1-1* (Blecker *et al.*, 1988), the SA-insensitive mutant *npr1* (Cao *et al.*, 1994), the enhanced disease susceptibility mutant, *eds1* (Parker *et al.*, 1996), and *ndr1*, which compromises signalling by *CC NBS LRR* genes (Century *et al.*, 1995; Aarts *et al.*, 1998). The expression of the SA-dependent *PR1* and JA-dependent *PDF1.2* genes were subsequently studied by northern blot analysis in the resulting panel of *adr2 adr2* double mutants (Fig. 7a).

The level of *PR1* gene expression in *adr2 adr2* plants was similar to that detected in SA-treated wild-type plants (Fig. 7a). Furthermore, significant *PR1* transcript accumulation in *adr2 adr2* plants was also observed in the presence of *ndr1*, *etr1* or *coi1*. However, *PR1* expression was reduced in *adr2 adr2 eds1* plants and absent in *adr2 adr2 nahG* or *adr2 adr2 npr1* plants. There was a small but significant accumulation of

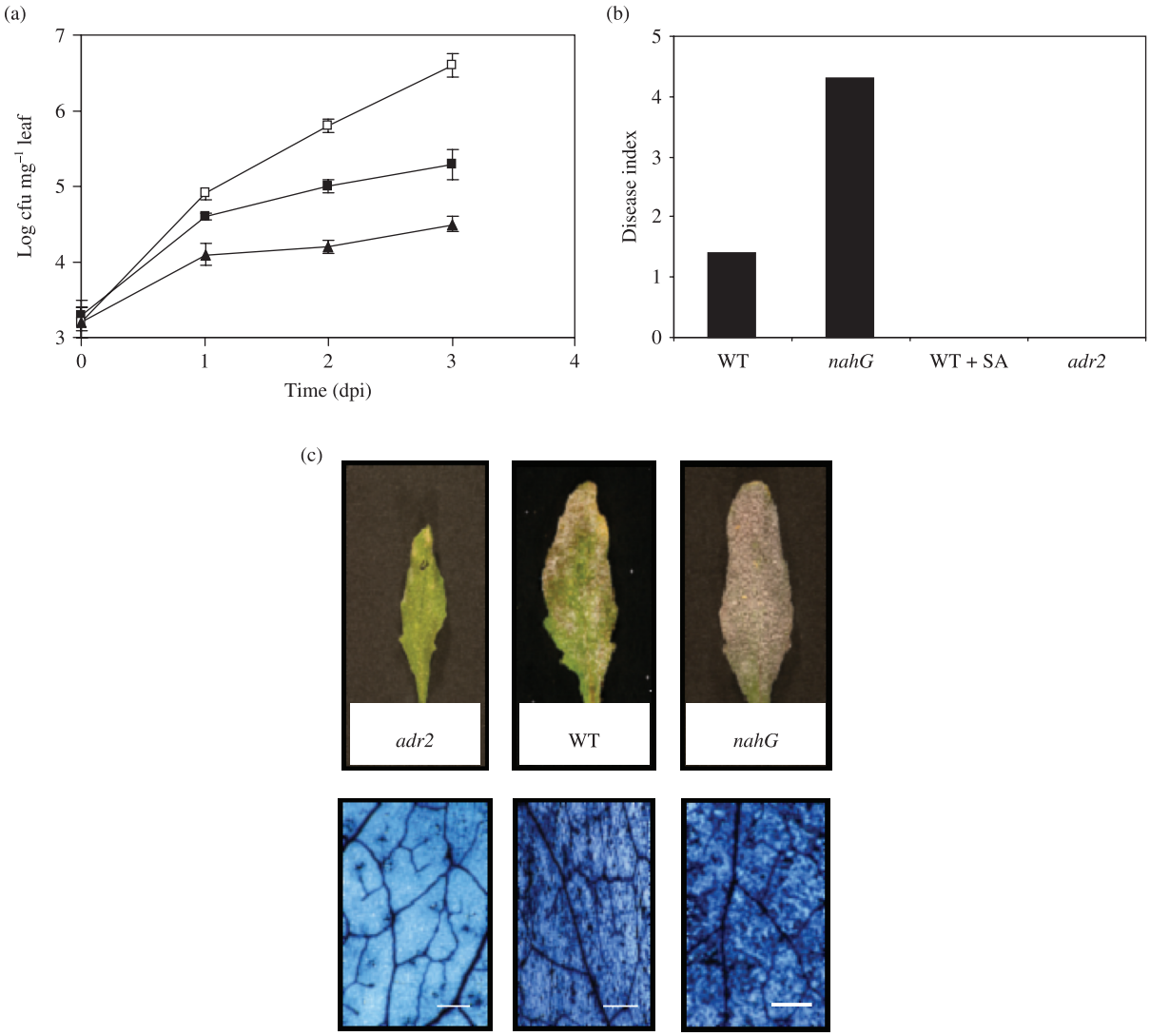

Fig. 6 The *adr2* line exhibits heightened pathogen resistance. (a) Titre of virulent *Pst*DC3000 in *adr2* plants compared with wild-type. Leaves of corresponding plants were infiltrated with 0.0002 OD_{600} bacterial suspension in 10 mM $MgCl_2$. Leaf samples were assayed for bacterial growth at the indicated time-points expressed as days postinfection (dpi). Bacterial growth is represented as log cfu mg^{-1} leaf tissue. The experiment was repeated three times with similar results. Error bars represent the standard error of the mean. Closed squares, wild type; open squares, *nahG*; triangles, *adr2*. (b) Growth of the oomycete *Hyaloperonospora arabidopsis* Noco2 on *adr2* leaves compared with wild-type. Plants were sprayed with an oomycete suspension at a density of 10^5 spores ml^{-1}. Plants were examined for *H. arabidopsis* growth at 7 dpi and a disease index was assigned for each group of plants. Each group consisted of 10 plants. (c) Growth of the biotrophic fungus *Golovinomyces cichoracearum* UED1 on *adr2,* wild-type and *nahG* plants, as revealed by the staining of fungal structures with Trypan blue. Bars, 10 μm.

PDF1.2 transcripts in *adr2 adr2* plants and this was strikingly increased in the *adr2 adr2 nahG* line. By contrast, *PDF1.2* expression was abolished in *adr2 adr2* plants in the presence of *coi1*, *ndr1* and *etr1*.

We also monitored lesion development in this set of double mutants. The depletion of SA by *nahG* or the presence of *npr1* or *eds1* in *adr2* plants abolished lesion formation (data not shown). By contrast, *ndr1*, *coi1* or *etr1* failed to affect lesion formation in the *adr2* line. This panel of double mutants was also challenged with *H. arabidopsis*. As expected, *adr2 adr2* plants exhibited significant resistance against this pathogen (Fig. 7b). Interestingly, *ndr1* did not affect resistance against *H. arabidopsis* in *adr2 adr2* plants. By contrast, the presence of *npr1* diminished *adr2*-mediated resistance to a level similar to that expressed by wild-type plants. However, the presence of either *nahG* or *eds1* supported strikingly increased growth of *H. arabidopsis* in the *adr2 adr2* line. Resistance against this pathogen in *adr2 adr2* plants was not compromised by *coi1* or *etr1* (data not shown).

Mapping and cloning of *ADR2*

In order to determine the mode of inheritance of the *adr2* mutation, homozygous *adr2* plants were backcrossed to the

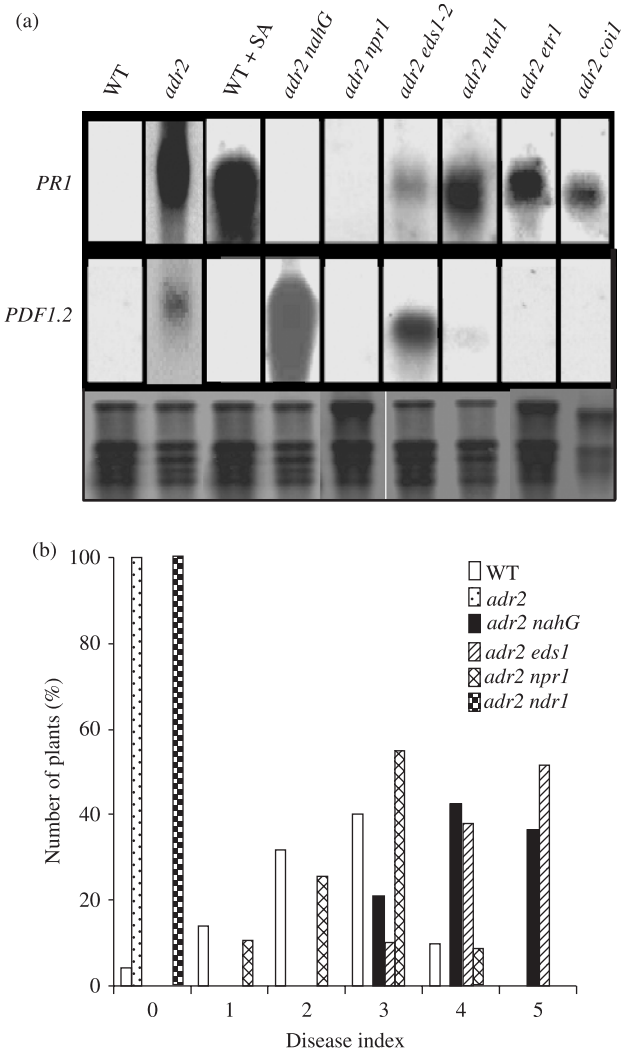

Fig. 7 Impact of *nahG*, *npr1*, *ndr1*, *eds1*, *etr1* and *coi1* on *adr2*-mediated *PR1* and *PDF1.2* gene expression and resistance against *Hyaloperonospora arabidopsis*. (a) RNA was isolated from the indicated genotypes and subjected to northern blot analysis with *PR1* and *PDF1.2* probes. The RNA in each lane was also stained with methylene blue as a control for equal loading and RNA transfer. (b) Infection of the given *adr2* double mutants with *H. arabidopsis*. Infection was carried out by spraying a 1×10^5 conidiospore suspension of *H. arabidopsis* and assaying for pathogen growth at 7 dpi.

wild type. The characteristic *adr2* phenotype, HR-like lesions and H_2O_2 accumulation were observed in all F_1 progeny, suggesting *ADR2* was a dominant mutation. Moreover, in F_2 progeny *adr2* segregated in a classic Mendelian fashion of 3 : 1 (32/8) ($\chi^2 = 0.032$, $P > 0.5$). Hence, the *adr2* mutation segregates as a dominant, monogenic trait. The *adr2* gene was mapped by crossing *adr2 adr2* plants (in Col-0) to the L*er* accession. An approximate map position for *adr2* was obtained by bulked segregant analysis (Lukowitz *et al.*, 2000). This analysis showed that the *adr2* gene lies on chromosome 1 between the SSLP markers nga111 and nga128.

The dominant *adr2*-associated traits cosegregated with insensitivity to the ammonium glufosinate herbicide encoded within the T-DNA. Sequences flanking this T-DNA insert were recovered by TAIL-PCR (Singer & Burke, 2003). By interrogating the TAIR database (http://www.arabidopsis.org), the T-DNA insert was found to reside on chromosome 1 between two *TIR NBS LRR* genes (*At1g56520* and *At1g56510*), with a third *TIR NBS LRR* (*At1g56540*) approx. 6 kb away from the T-DNA insert beyond *At1g56520* (Fig. 8a). Expression analysis of genes around the T-DNA tag by reverse-transcriptase PCR revealed that only the three *TIR NBS LRR* genes were ectopically overexpressed in *adr2* plants (Fig. 8b). To identify *ADR2* we employed a transgenic reconstitution approach. Thus, full-length cDNAs were generated for each of these genes and their integrity confirmed by DNA sequencing. The resulting cDNA sequences were subsequently cloned behind the CaMV*35S* promoter and the constructs generated were transformed individually into wild-type Col-0 plants using floral dip transformation (Clough & Bent, 1998). Overexpression of *At1g56510* but not either *At1g56520* or *At1g56540* reconstituted the *adr2* phenotype in T1 and T2 plants. To confirm that basal disease resistance was increased, plants were challenged with virulent *Pst*DC3000 and scored for pathogen titre over time. Overexpression of *At1g56510* conveyed cell death development and robust resistance against attempted *Pst*DC3000 infection in a similar fashion to *adr2* (Fig. 9a). Furthermore, increased expression of this gene also resulted in enhanced protection against *G. cichoracearum* UED1 (Fig. 9b). Collectively, these findings imply that *At1g56510* is *ADR2*.

Discussion

We have employed activation tagging in an *Arabidopsis* line containing a *PR1::LUC* transgene to uncover genes whose enhanced expression leads to the establishment of broad-spectrum disease resistance. One mutant, designated *adr2*, has been described in this report. The *adr2* mutant conveyed significant constitutive expression of both LUC activity and the endogenous *PR1* gene. *adr2* is a dominant, gain-of-function, monogenic mutation that conveys spreading HR-like lesions, accumulation of H_2O_2 and auto-fluorescent phenolic compounds. Significantly, *adr2* plants also exhibit broad-spectrum disease resistance.

Several *Arabidopsis* mutant classes displaying constitutive lesion formation have previously been described including *lsd*, *acd*, *cpr5* and *cpr6* mutants (Dietrich *et al.*, 1994; Greenberg *et al.*, 1994; Weymann *et al.*, 1995; Bowling *et al.*, 1997; Clarke *et al.*, 1998). Of the previously described lesions mimics, only *acd6*, *lsd2*, *lsd4*, *lsd6* and *lsd7* are dominant. Notably, *acd6* leaves display a few punctate cell death patches as opposed to the uniformly-distributed mosaic-like lesions that are associated with the *adr2* phenotype. Unlike *adr2*, the spontaneous lesion formation phenotypes associated with the *lsd2* and *lsd4* mutations have previously been shown to be SA-independent

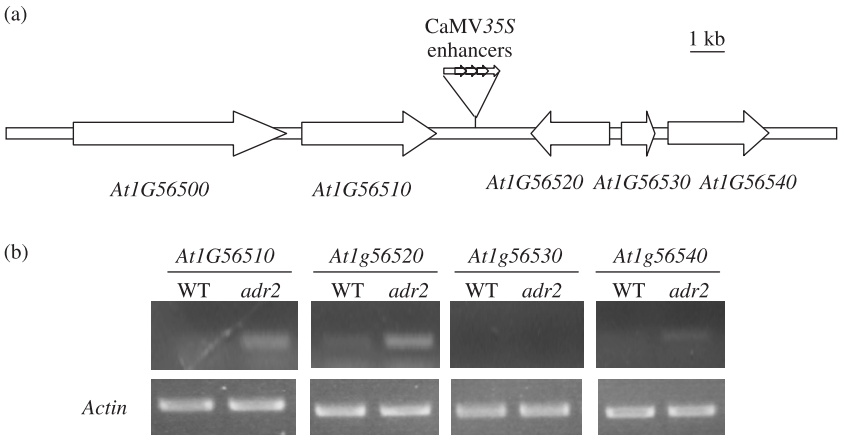

Fig. 8 Position of the activation tag in *adr2* plants. (a) Schematic showing the location of the T-DNA insertion, containing four copies of the *35S* enhancer sequence, within the *adr2* line. Adjacent genes along with their corresponding identification numbers are shown. (b) Reverse transcriptase polymerase chain reaction analysis of the indicated genes in the given plant genotypes.

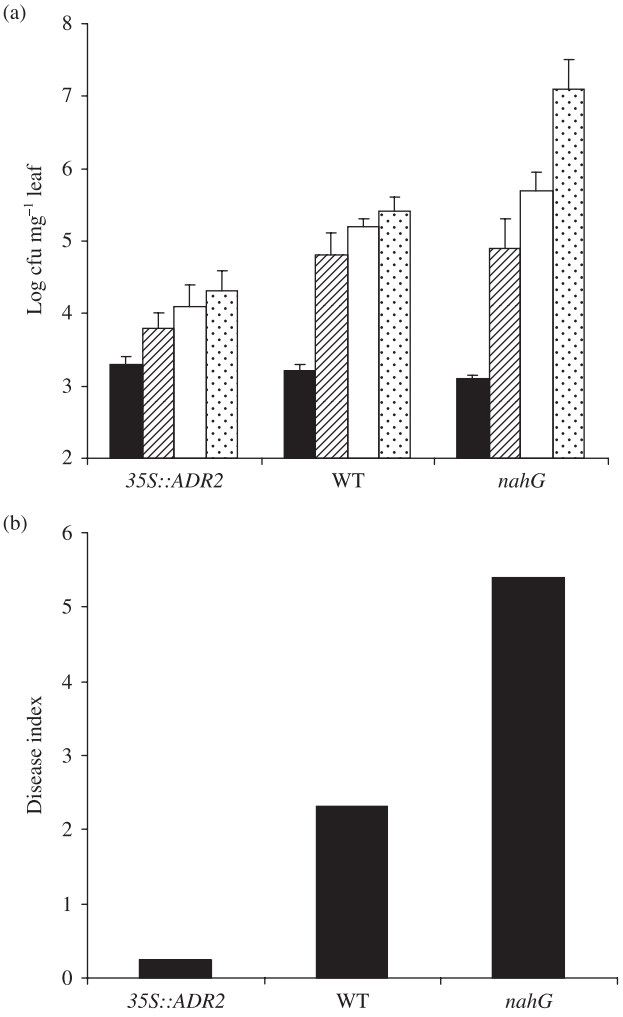

Fig. 9 CaMV*35S*::*At1g56510* plants exhibit increased disease resistance. (a) Plants containing a CaMV*35S*::*At1g56510* transgene show heightened resistance against virulent *Pst*DC3000. Closed, hatched, open and dotted bars indicate bacterial titre at 0, 2, 3 and 4 d, respectively, postinoculation in the indicated genotypes. (b) The given plant lines were challenged with *Golovinomyces cichoracearum* UED1 and scored for disease resistance. The experiments were repeated twice with similar results. Error bars represent the standard error of the mean.

(Hunt *et al.*, 1997). Moreover, when compared with *adr2*, both *lsd2* and *lsd4* display distinct HR-like lesion phenotypes and distribution. Therefore, despite the fact that no map position has previously been assigned to these mutations, *adr2* is unlikely to be allelic to either *lsd2* or *lsd4*. By contrast, unlike *lsd2* and *lsd4*, lesion formation on *lsd6* and *lsd7* leaves has previously been reported to be SA-dependent (Weymann *et al.*, 1995). The *lsd7* phenotype is associated with very small lesions that became evident when visualized microscopically by Trypan blue staining. Moreover, *lsd7* has been shown not to be linked to chromosome I (Weymann *et al.*, 1995). Hence, we conclude that *adr2* is also not allelic to *lsd7*. The *lsd6* phenotype is characterized by spontaneous formation of punctate necrotic lesions that are not affected by daylength. Interestingly, the spontaneous lesion formation on *lsd6* leaves was blocked under high humidity. The *lsd6* mutation has been mapped to the lower arm of chromosome I, within an *c.* 25 cM region, between the SSLP markers nga111 and nga128 (Weymann *et al.*, 1995). However, *adr2* was not found to reside in this region. Thus, *adr2* is also not allelic to *lsd6*. Together, our data imply that mutations in *ADR2* leading to a spreading lesion phenotype have not been described previously.

The *adr2* line was found to exhibit broad-spectrum resistance against two biotrophic microbial pathogens, *H. arabidopsis* Noco2 and *G. cichoracearum* UED1 and a hemibiotrophic pathogen, *Pst*DC3000. Depletion of SA blunted *PR1* expression and abolished resistance against *H. arabidopsis* Noco2, suggesting that SA accumulation is essential for *adr2*-mediated resistance against these pathogens. Moreover, *npr1* or *eds1* also abolished *PR1* gene expression in *adr2* plants. Functional EDS1 but not NPR1 was also required for *adr2*-mediated resistance against *H. arabidopsis* Noco2, as resistance was dramatically reduced in the respective double mutants. NPR1 was also found to be dispensable for *H. arabidopsis* Noco2 resistance in the *cpr1*, *cpr5* and *cpr6* mutants (Bowling *et al.*, 1994, 1997; Clarke *et al.*, 1998).

Interestingly, there was a small but significant accumulation of *PDF1.2* transcripts in *adr2 adr2* plants, suggesting that *adr2* also weakly activates expression of this JA/ET-dependent

marker gene. This was unexpected because SA accumulation is thought to routinely suppress JA signalling (Petersen *et al.*, 2000; Glazebrook, 2005). In the presence of either *coi1* or *etr1* this *adr2*-mediated expression of *PDF1.2* was abolished, suggesting that *adr2* activates both weak ET- and JA-dependent signalling. Similar observations were made for the *adr1* mutant line which also expressed *PDF1.2* in addition to *PR1* (Grant *et al.*, 2003). Unexpectedly, this low level of *PDF1.2* expression is also lost in *adr2* plants in the absence of NPR1 function. NPR1 has previously been proposed to suppress JA signalling (Spoel *et al.*, 2003), at least in lines without *adr2*. Strikingly, in *adr2 adr2 nahG* and *adr2 adr2 eds1* plants the accumulation of *PDF1.2* transcripts is markedly increased relative to the *adr2 adr2* line. This further supports the idea that SA signalling is antagonistic to JA signalling. Despite the relatively low level of constitutive *PDF1.2* expression *adr2 adr2* plants do not exhibit increased resistance against the necrotrophic pathogen *B. cinerea*. Engagement of the defence response following attempted infection by this class of pathogen is thought to be dependent upon expression of JA- and ET-dependent defence genes (Thomma *et al.*, 1998). However, the magnitude of JA signalling marked by *PDF1.2* expression in *adr2 adr2* plants maybe insufficient to convey protection against *B. cinerea*. Furthermore, *adr2* triggered *COI1*- and *ETR1*-dependent *PDF1.2* gene expression was unnecessary for resistance against *H. arabidopsis* Noco2 because the growth of this pathogen on *adr2 adr2* plants was not affected by either *coi1* or *etr1*.

The *adr2* line accumulates H_2O_2 and exhibits a marked increase in MAPK activity. This is a common plant response to a variety of different biotic and abiotic stresses (Inzé & van Montagu, 1995). In many eukaryotes, the transduction of oxidative signals is controlled by protein phosphorylation involving MAPKs. These MAPKs and their immediate upstream activators, MAPKKs and MAPKKKs, constitute a functionally interlinked MAPK cascade (Jonak *et al.*, 1994). However, our current knowledge as to the precise role(s) of MAPK signalling in disease resistance remains rather limited. Elevation of MAPK activity has been detected in plants after exposure to various stimuli including AVR proteins (Romeis *et al.*, 1999), H_2O_2 (Grant *et al.*, 2000), flg22, a peptide derived from bacterial flagellin (Asai *et al.*, 2002), and SA (Kumar & Klessig, 2000). Our findings indicate that *adr2* constitutively activates two MAPK activities of 46 kDa and 48 kDa. We speculate that this constitutive MAPK activity detected in *adr2* plants is possibly initially cued by the high H_2O_2 concentrations in this line, which subsequently leads to the synthesis and accrual of SA. This key plant immune activator may then govern a positive feedback loop that promotes further H_2O_2 production during the defence response. In this context, SA has been shown to potentiate the oxidative burst during the development of disease resistance (Shirasu *et al.*, 1997). Thus, MAPK signalling in *adr2* plants may be engaged by ROIs and subsequently amplified by SA.

The insertion of the T-DNA activation tag in *adr2* plants was found to reside between two *TIR NBS LRR* genes (*At1g56520* and *At1g56510*), with a third *TIR NBS LRR* (*At1g56540*) approx. 6 kb away from the T-DNA insert beyond *At1g56520*. A RT-PCR analysis revealed that all these *TIR NBS LRR* genes were ectopically overexpressed in *adr2* plants, suggesting the *35S* enhancers present within the inserted T-DNA increased the basal expression levels of these genes. Thus, implying that the *adr2* phenotype is the result of overexpression of *At1g56520*, *At1g56510* or *At1g56540*, or a combination of these three *TIR NBS LRR* genes. Furthermore, this idea is also supported by the double mutant analysis because while *adr2 adr2 ndr1* plants exhibited broad-spectrum disease resistance the *adr2 adr2 eds1* line did not. NDR1 is known to be essential for defence signalling following pathogen recognition by CC NBS LRR proteins but is dispensable for defence signalling activated by *TIR NBS LRR* gene products. By contrast, EDS1 is essential for defence signalling following pathogen recognition by TIR NBS LRR proteins but is dispensable for CC NBS LRR-mediated disease resistance (Aarts *et al.*, 1998). Therefore, the loss of broad-spectrum disease resistance in *adr2 adr2 eds1* but not *adr2 adr2 ndr1* plants is consistent with the notion that overexpression of a *TIR NBS LRR* gene is responsible for this phenotype. Our findings from transgenic reconstitution experiments identified *At1g56510* as *ADR2*.

A prior example of *TIR NBS LRR* overexpression resulting in defence activation has been reported previously (Stokes *et al.*, 2002). Here, the metastable epigenetic variant *bal* led to overexpression of a single *TIR NBS LRR* gene (*At4g16890*) from a cluster of *R* genes on chromosome 4. This resulted in plants of reduced stature that showed constitutive activation of SA-dependent defence responses (Stokes *et al.*, 2002). However, spreading lesion development was not associated with this line. Also, in an Ethyl Methane Sulphonate (EMS) screen for suppressors of *npr1-5*, the *ssi4* mutation was uncovered which resulted in increased expression of *SSI4*, a *TIR NBS LRR* gene (Shirano *et al.*, 2002). The *ssi4* line exhibited lesion development, constitutive activation of SA signalling and broad-spectrum disease resistance. Interestingly, *ssi4* was found to be a substitution within the NBS domain of *SSI4* and furthermore overexpression of *SSI4* in transgenic plants did not recapitulate the *ssi4* phenotype. In contrast to *bal*, this mutation was therefore thought to cause activation of SSI4 resulting in the subsequent engagement of SA-dependent signalling that ultimately led to the increased expression of *SSI4*, which was shown to be a SA-responsive gene. Therefore, only the ectopic expression of a subset of *TIR NBS LRR* genes is likely to result in the activation of plant defence responses.

Collectively, our data suggests that overexpression of *At1g56510* triggers an *EDS1*, *NPR1* and SA-dependent defence signalling pathway that establishes broad-spectrum disease resistance against biotrophic pathogens in *Arabidopsis*. This may occur because an increase of At1g56510 might titrate out a guard protein that ordinarily sequesters the defence

signalling function of this TIR-NBS-LRR protein in the absence of pathogen-derived cues. A biological manifestation of this phenomenon is the recent demonstration that intraspecies and possibly interspecies hybrid necrosis may be underpinned by specific epistatic interactions that trigger R-protein signalling (Bomblies *et al.*, 2007). This is also thought to result from a breakdown in the interaction between a given NBS LRR and its guard protein.

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